Abstract

Water availability is a major limiting factor on plant growth and productivity. Considering that Eucalyptus spp. are among the few plant species able to produce both isoprene and monoterpenes, experiments were designed to investigate the response of isoprene emission and isoprenoid concentrations in Eucalyptus citriodora saplings exposed to decreasing fraction of transpirable soil water (FTSW). In particular, this study aimed to assess: (a) the kinetic of water-stress-induced variations in photosynthesis, isoprene emission, and leaf isoprenoid concentrations during progressive soil water shortage as a function of FTSW; (b) the ultradian control of isoprene emission and photosynthesis under limited soil water availability; and (c) the optimum temperature sensitivity of isoprene emission and photosynthesis under severe water stress. The optimum temperature for isoprene emission did not change under progressive soil water deficit. However, water stress induced a reallocation of carbon through the MEP/DOXP pathway resulting in a qualitative change of the stored isoprenoids. The ultradian trend of isoprene emission was also unaffected under water stress, and a similar ultradian trend of stomatal and mesophyll conductances was also observed, highlighting a tight coordination between diffusion limitations to photosynthesis during water stress. The kinetics of photosynthetic parameters and isoprene emission in response to decreasing FTSW in E. citriodora are strikingly similar to those measured in other plant functional types. These findings may be useful to refine the algorithms employed in process-based models aiming to precisely up-scale carbon assimilation and isoprenoid emissions at regional and global scales.

Key words: Carbon assimilation, diffusive limitations, diurnal variability, drought kinetics, high temperature, isoprenoids.

Introduction

Plants invest part of the assimilated carbon to produce various isoprenoids (Dudareva et al., 2006). Isoprenoid formation is photosynthesis-dependent in non-stressed plants via de novo synthesis of dimethylallyl diphosphate in the chloroplast (Loreto et al., 1996; Rosenstiel et al., 2002; Sharkey et al., 2008; Loreto and Schnitzler, 2010). However, processes regulating photosynthesis and isoprenoid metabolism in plants are differently affected by water stress (Brilli et al., 2007;
control of isoprene emission as well as of transport limitations to photosynthesis under conditions of reduced water availability.

**Materials and methods**

**Plant material and experimental design**

*E. citriodora* is an evergreen tree species native of Australia. Because of its wide range of adaptation and ecological amplitude, *E. citriodora* is widely grown in arid areas of Pakistan as part of fuel-wood plantations. Two-year-old *E. citriodora* saplings were propagated from mature trees collected in the Sindh province (Pakistan) and grown in 10 dm³ pots, filled with a mixture of 50% commercial soil and 50% sand, in a greenhouse in Monterontondo Scalo (Rome, Italy, 42° 04’ N 12° 36’ E). All the saplings were regularly watered and fertilized with Hoagland solution once a week in order to supply mineral nutrients at free access rate (Magnani et al., 1996; Centritto et al., 1999).

Two different water stress experiments were performed. The first experiment was carried out at the beginning of the summer, whereas the second one was performed at the onset of the following autumn. In both experiments, on the afternoon prior to initiate the water stress experiments, all plants were fully irrigated and the excess water was allowed to drain overnight. After draining, the pots were weighed to 1-g precision on a digital balance (model Q532A, Sartorius Instrumentation, Göttingen, Germany) to determine the weight at pot water capacity (Initial potweight). Each pot was then enclosed in a plastic bag that was tied around the stem to prevent soil evaporation. Ten plants were water-stressed by withholding water, while other 10 saplings continued to be well watered to pot capacity in order to represent control plants. Water stress development was followed and parameterized by daily recording of the pot weight to be finally expressed as fraction of transpirable soil water (FTSW) (Sinclair and Ludlow, 1986; Brilli et al., 2007). The physiological lower limit of available soil water was defined as the FTSW at which stomatal conductance approached zero (i.e. soil water decreased to a level where there was no longer water available to support transpiration) (Sinclair and Ludlow, 1986). Once this level was achieved, the water-stressed pots were weighed to determine the final pot weight (Final potweight). Thus, every morning, during the drought stress cycle, all the plastic bags were unwrapped to weigh the water-stressed saplings (Daily potweight) and to water the control plants. Then, the FTSW was calculated for each single pot as:

\[
\text{FTSW} = \frac{\text{Daily potweight} - \text{Final potweight}}{\text{Initial potweight} - \text{Final potweight}}.
\]

The first water stress cycle lasted 25 days. After reaching the FTSW endpoint (Final potweight), all water-stressed saplings were rewettered and maintained at full pot capacity over a 7-day recovery period (DAR).

All gas exchange measurements were made at the saturating photosynthetic photon flux density (PPFD) of 1000 µmol m⁻² s⁻¹. PPFD was measured with a LI-COR quantum meter (Lincoln, NE, USA). The measurements were carried out with two different infrared gas analyser systems, as specified below. Leaves enclosed in the gas exchange cuvettes of the two systems were exposed to a 500 ml min⁻¹ flow of synthetic air made by mixing the air components from pure cylinders. The final reconstituted mixture contained 80% N₂, 20% O₂, 380 µmol mol⁻¹ CO₂, and no ozone, isoprenoids, or other trace gases and contaminants.

### First water stress experiment

Photosynthesis (A), stomatal conductance (gₛ), internal CO₂ concentration (Cᵢ), and isoprenoid concentrations were measured between 11:00 and 13:00 h during the water stress cycle and recovery period. A round portion (6cm²) of leaves was clamped in a custom-built gas-exchange cuvette coated with Teflon and with glass windows on both sides. Water from a thermostated water bath was circulated...
through the aluminum body of the cuvette to control the leaf temperature. Leaf temperature was set at 30 °C and was measured by a thermocouple firmly appressed to the abaxial leaf surface. The relative humidity in the leaf cuvette ranged between 45 and 50%. CO₂ and H₂O exchange were measured at steady conditions by a LI-7000 CO₂/H₂O infrared analyser (LI-COR).

The diurnal variations in J, gs mesophyll conductance (gₘ), and isoprene emissions were measured in well-watered and water-stressed plants with a portable gas exchange system (LI-6400, LI-COR) equipped with the 6400-06 PAM-2000 adapter cuvette to hold a fibre probe of a fluorescence system (MiniPAM, Walz, Effeltrich, Germany). The tip of the optic fibre of the MiniPAM was inserted in one corner of the cuvette window at an angle of 45° to the surface. The optic fibre could be placed about 1 cm from the leaf without shading it (Loreto et al., 2003; Shi et al., 2006). To reduce diffusion leaks through the chamber gaskets, the CO₂ and H₂O gradients between the in-chamber air and pre-chamber air were minimized by enclosing the whole leaf chamber in a polyvinyl fluoride bag supplied with the exhaust air from the infrared gas analyser as described by Rodeghiero et al. (2007). Measurements were carried out at the leaf temperature of 30 °C, and at relative humidity in the leaf cuvette ranging between 45 and 50%. Gas exchange parameters and chlorophyll fluorescence yield were measured simultaneously. The fluorescence yield was measured following a saturating pulse (10000 µmol m⁻² s⁻¹) of white light. The electron transport rate was calculated by fluorescence as described by Genty et al. (1989), Γ* (the CO₂ compensation point between photosynthesis and photorespiration) used in the gas exchange algorithm was calculated with the Rubisco specific factor estimated for woody evergreen by Galmés et al. (2005). Because Γ* is a remarkably conservative parameter (Harley et al., 1992), it was assumed that the value used in the gas exchange algorithm did not affect the estimation of gₘ. Whereas, dark respiration, which was taken as a proxy for light respiration (Centritto et al., 2009, 2011a), was measured after maintaining leaves in darkness for 10 min. Then, gₘ was calculated using variable J method, as described by Harley et al. (1992). The occurrence of stomatal patchiness could not be estimated, which might have affected the Cᵢ calculation and, in turn, the accuracy of gₘ measurements in severely stressed plants; hence, it was impossible to rule out the occurrence of measurement errors (Flexas et al., 2008). However, Centritto et al. (2009) have compared the variable J method with carbon isotope discrimination in recently synthesized sugars to calculate gₘ in water-stressed rice and found that the two methods yielded congruent estimations of gₘ, confirming the reliability of the variable J method even in drought-stressed samples.

Following gas exchange measurements, isoprene emission was online detected by connecting the cuvette outflow to a proton transfer reaction mass spectrometer (PTR-MS) (Ionicon, Austria). Details on the theory and practice of the PTR-MS technique were reported by Lindinger et al. (1998) whereas applications of this technique in cuvette studies jointly with other gas-exchange measurements are described by Tholl et al. (2006). Before each set of measurements, the PTR-MS was calibrated against an isoprene gaseous standard (70 ml l⁻¹, Rivoira, Milan, Italy).

Isoprenoid concentration was measured as first reported by Loreto et al. (1998) and expressed on a leaf area basis. Leaf planar area was measured with a leaf area meter (LI-3000, LI-COR). After assessing the area, two leaves per plant (4 or 5 plants per treatment) were first plunged in liquid nitrogen and then ground to powder, and subsequently transferred to a test tube. A flow of helium (200ml min⁻¹) was then passed through the test tube, and released isoprenoids were collected in a silica-coated cartridge packed with 200mg of Tenax (Markes International, UK). The silica coated cartridges were then analysed through a thermal desorber UNITY (Markes International) by using a gas chromatograph (GC-Agilent 6850, Agilent Technologies, Wilmington, DE, USA) equipped with a splitless injector and a HP-5MS capillary column (30 m in length, 250 µm i.d., and 0.25 µm film thickness) and coupled with a mass selective detector (MS-Agilent 5975C, Agilent Technologies). The GC-MS system was calibrated using gas standard for the target compounds (Rivoira). Then the concentration of different compounds was calculated by direct comparison with the peak area of the respective gaseous standard. Different compounds were identified via the NIST library provided with the GC/MS ChemStation software (Agilent). GC peak retention time was substantiated by analysis of parent ions and main fragments on the spectra.

### Second water stress experiment

The saplings which had already experienced water stress during the first experiment were water stressed again at the beginning of autumn, while the other saplings continued to grow in well watered conditions (i.e. 100% of FTSW). To measure gas exchange responses to short-term increase in temperature, a round portion (6cm²) of leaves was clamped in a custom-built gas-exchange cuvette coated with Teflon and with glass windows on both sides. As described above, leaf temperature was controlled by circulating water from a thermostated water bath through the aluminum body of the cuvette; CO₂ and H₂O exchange were measured using a LI-7000 CO₂/H₂O infrared analyser, whereas isoprene emission was detected on-line with a PTR-MS. The temperature of the clamped leaves in the gas-exchange cuvette was progressively increased and measurements were made at five different temperatures (30, 35, 40, 45, and 50 °C). Measurements were recorded when physiological parameters (A, gs, and gₘ emission) were at steady-state for about 15 min. The temperature was then again decreased to 30 °C and measurements were made again after 30 more minutes, 12h, and 24h.

### Statistical analysis

Data were tested using a simple factorial ANOVA (two-way maximum interactions) to determine the main effects of water treatment and temperature on all dependent variables measured. Where appropriate, the means of the different treatments were compared using Tukey's post-hoc test.

### Results

Net CO₂ assimilation (A) and stomatal conductance (gₛ) of *E. citriodora* plants responded to water deficit (Fig. 1A,B) only when the FTSW was lower than 40% (Table 1). Whereas, only at FTSW₅ intercellular CO₂ concentration (Cᵢ) was significantly lower than in control plants (Table 1), implying a well-functioning regulation of diffusional limitations of photosynthesis. However, there were a few Cᵢ values higher than in control plants at the FTSW endpoint (Fig. 1C), implying that photosynthesis was likely limited by biochemical factors. Isoprene emission (Fig. 1D) also decreased only slowly, and its reduction became statistically significant only when the FTSW was largely reduced (Table 1).

There were significant differences in the diurnal variations in A (P < 0.01), gₛ (P < 0.001), gₘ (P < 0.001), and isoprene emission (P < 0.001) in plants undergoing different levels of water stress (Fig. 2). In well-watered plants and treated plants in which the FTSW was 50%, A reached the maximum about 30 min after beginning of illumination and then slowly decreased (Fig. 2A). Among plants set up at FTSW₅, a trend was observed that was similar to the plants grown at higher FTSW, but the initial maximum after illuminating the leaves was 60–70% lower than in those plants. In plants at FTSW₅, A was almost completely inhibited and only a short burst was observed around 50 min after illumination. Diurnal variations
in \( g_s \) and \( g_m \) were very similar, also peaking 50–60 min after illumination and slowly decreasing during the following time-course (Fig. 2B,C). However, contrary to \( A \), significant daily reductions of \( g_s \) and \( g_m \) were observed in plants at FTSW 50 with respect to fully irrigated controls (Table 2). The two conductances were largely reduced at FTSW 25 and further inhibited at FTSW 5 (Table 2).

Illumination-induced isoprene emission much more slowly than \( A \), at all FTSWs (Fig. 2D). In well-watered controls, isoprene peaked twice, around 150 and 300 min after illumination, and slowly dropped after the second peak. Water stress apparently suppressed the first daily peak of isoprene emission. At FTSW 50 and FTSW 25, maxima isoprene emissions were recorded at or after 300 min of illumination. Isoprene was still emitted at measurable levels in severely stressed leaves (FTSW 5) (Table 2), but in this case a plateau emission was reached after 100 min and was maintained until around 300 min of illumination, after which the emission dropped.

Isoprenoid concentration was measured on a leaf area basis in order to avoid the likely water stress-induced effects on leaf mass, and to make these concentrations comparable to isoprene emission fluxes. A slight, non-significant increase in the total concentration of isoprenoids was observed at decreasing FTSW (Table 3). When looking at the single compounds, two major monoterpenes (citronellal and citronellol) and other minor compounds increased with decreasing FTSW. On the other hand, isopulegol largely dropped at relatively mild water stress (FTSW 50), and carvophyllene oxide was not detected at severe water stress (FTSW 5) (Table 3).

Exposure of control plants to rising temperatures led to a sustained decrease of \( A \) (Fig. 3A). This reduction had been
observed at 35 °C, and at 50 °C A was reduced by more than 50% with respect to 30 °C. The temperature-driven inhibition was transient and could be fully recovered 12 h after returning to 30 °C (Fig. 3A). In severely stressed leaves in which A was already strongly inhibited (i.e. at 10% or lower FTSW), the impact of rising temperatures was lower or absent. At FTSW10 a transient stimulation of A was observed immediately upon returning to 30 °C. Isoprene emission showed an optimum at ~45 °C in control and in severely stressed plants (FTSW10), but when FTSW approached the end point (FTSW5), the stimulating effect of temperature over isoprene emission was mostly lost, and optimum emission was reached at 40 °C (Fig. 3B). Isoprene emission dropped at 50 °C irrespective of A (µmol m⁻² s⁻¹).

Table 2. Photosynthesis (A, µmol m⁻² s⁻¹), stomatal conductance (gₛ, mol m⁻² s⁻¹), mesophyll conductance (gₘ, mol m⁻² s⁻¹), and isoprene emission (nmol m⁻² s⁻¹) as a function of fraction of transpirable soil water (FTSW) in Eucalyptus citriodora saplings at the end of the time course measurements shown in Fig. 2. Data are means of four leaves ± 1 SEM. Superscript letters in the same column indicate significant differences at P < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>A (µmol m⁻² s⁻¹)</th>
<th>gₛ (mol m⁻² s⁻¹)</th>
<th>gₘ (mol m⁻² s⁻¹)</th>
<th>Isoprene emission (nmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTSW100</td>
<td>9.67 ± 0.49a</td>
<td>0.132 ± 0.007b</td>
<td>0.091 ± 0.009b</td>
<td>14.88 ± 0.75b</td>
</tr>
<tr>
<td>FTSW50</td>
<td>8.70 ± 0.44a</td>
<td>0.090 ± 0.007b</td>
<td>0.078 ± 0.009b</td>
<td>10.77 ± 0.54a</td>
</tr>
<tr>
<td>FTSW25</td>
<td>1.76 ± 0.09b</td>
<td>0.019 ± 0.009b</td>
<td>0.010 ± 0.001a</td>
<td>7.66 ± 0.39b</td>
</tr>
<tr>
<td>FTSW5</td>
<td>0.83 ± 0.04a</td>
<td>0.004 ± 0.001a</td>
<td>0.007 ± 0.001a</td>
<td>5.36 ± 0.27a</td>
</tr>
</tbody>
</table>

Table 3. Leaf isoprenoid concentrations on a leaf area basis (µg cm⁻²) in Eucalyptus citriodora plants measured during and after the water stress cycle. Measurements were made at 100% (FTSW100), 50% (FTSW50), and 5% (FTSW5) fractions of transpirable soil water (FTSW) respectively and 7 days after relief of water stress (DAR). Data are means of 4 or 5 plants (two leaves per plant) ± 1 SEM. Superscript letters in the same line indicate significant differences at P < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>FTSW100</th>
<th>FTSW50</th>
<th>FTSW5</th>
<th>7 DAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-pinene</td>
<td>0.013 ± 0.002a</td>
<td>0.012 ± 0.003b</td>
<td>0.013 ± 0.003a</td>
<td>0.013 ± 0.003a</td>
</tr>
<tr>
<td>Isopulegol</td>
<td>0.048 ± 0.009a</td>
<td>0.043 ± 0.005a</td>
<td>0.097 ± 0.021a</td>
<td>0.065 ± 0.008a</td>
</tr>
<tr>
<td>Citronellal</td>
<td>2.678 ± 0.272a</td>
<td>3.281 ± 0.135b</td>
<td>3.400 ± 0.286b</td>
<td>3.073 ± 0.300ab</td>
</tr>
<tr>
<td>Citronellic</td>
<td>0.137 ± 0.009a</td>
<td>0.178 ± 0.020b</td>
<td>0.168 ± 0.010a</td>
<td>0.152 ± 0.015ab</td>
</tr>
<tr>
<td>Citronelic acid</td>
<td>0.018 ± 0.005a</td>
<td>0.021 ± 0.003a</td>
<td>0.019 ± 0.006a</td>
<td>0.041 ± 0.007ab</td>
</tr>
<tr>
<td>Citronellol</td>
<td>0.041 ± 0.009a</td>
<td>0.021 ± 0.004a</td>
<td>0.023 ± 0.007a</td>
<td>0.041 ± 0.009a</td>
</tr>
<tr>
<td>Citronellic acid</td>
<td>0.041 ± 0.009a</td>
<td>0.021 ± 0.004a</td>
<td>0.023 ± 0.007a</td>
<td>0.041 ± 0.009a</td>
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<tr>
<td>Citronellil acetate</td>
<td>0.041 ± 0.009a</td>
<td>0.021 ± 0.004a</td>
<td>0.023 ± 0.007a</td>
<td>0.041 ± 0.009a</td>
</tr>
<tr>
<td>Caryophyllene</td>
<td>0.074 ± 0.017a</td>
<td>0.077 ± 0.021a</td>
<td>0.102 ± 0.026a</td>
<td>0.081 ± 0.019a</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>0.017 ± 0.004a</td>
<td>0.021 ± 0.002a</td>
<td>0.000a</td>
<td>0.000a</td>
</tr>
<tr>
<td>Others</td>
<td>0.040 ± 0.005a</td>
<td>0.112 ± 0.011a</td>
<td>0.033 ± 0.003a</td>
<td>0.034 ± 0.004a</td>
</tr>
<tr>
<td>Total</td>
<td>3.505 ± 0.186a</td>
<td>3.767 ± 0.141a</td>
<td>3.850 ± 0.159a</td>
<td>3.499 ± 0.176a</td>
</tr>
</tbody>
</table>
the stress level. When temperature was adjusted back to 30 °C, isoprene emission always recovered to the values measured before the temperature increase, but the recovery was faster in control leaves (12h) than in water-stressed leaves (36h).

Recovery of isoprene emission after rewatering of water-stressed *E. citriodora* saplings was fast and complete. The emission reached the pre-stress levels one day after rewatering and maintained the same emission for 7 days (Fig. 3). In contrast, $A$ and $g_s$ recovered more slowly, being less than 50% than in pre-stressed leaves after 1 day of rewatering. A complete recovery was observed after 3 and 7 days of rewatering for $g_s$ and $A$, respectively (Fig. 4).

**Discussion**

In the experiment performed at the beginning of summer, there was a slight but continuous decreasing trend in isoprene emission as water stress increased that resulted in a significant inhibition in isoprene emission when the stress became severe (Fig. 1D). This observation corroborates previous reports showing that isoprene biosynthesis, which is possibly supported by increasing contributions of extra-chloroplastic carbon sources under progressive water stress (Brilli *et al*., 2007), is resistant to water stress and, consequently, becomes uncoupled from photosynthesis under drought (Tingey *et al*., 1981; Sharkey and Loreto, 1993; Fang *et al*., 1996; Brilli *et al*., 2007; Centritto *et al*., 2011a).

*E. citriodora* displays similar kinetics of isoprene emission in response to FTSW to those observed in *Populus alba* (Brilli *et al*., 2007), *Populus nigra* (Centritto *et al*., 2011a), and *Syzygium cumini*, a sclerophyllous, evergreen tropical tree (M. Centritto and T. Mahmood, unpublished data). This may indicate that the inherent isoprene response to equivalent levels of soil water available for transpiration is similar across plants characterized by different functional traits (i.e. mesophyllous leaves and leaves with different degrees of sclerophyll) and by different volatile biosynthesis (eucalypts, unlike poplars, are also strong monoterpenes producers).

More interestingly, these results highlighted that water stress to some extent affects the ultradian regulation of isoprene emission (Fig. 2D), and that peak emission, shown ~150 minutes after inducing light conditions, was lost in water-stressed plants. In addition, the ultradian fluctuation of isoprene emission did not match the time-course variation in photosynthetic kinetics irrespective of the water stress level, because its induction emission was slower than that of photosynthesis and the emission trend remained steady longer than that of photosynthesis (Fig. 2A). The delayed peak of isoprene emission with respect to that shown by photosynthesis after illumination may have been likely caused by substrate limitation of ISPS activity as the dimethylallyl diphosphate pool undergoes to ultradian variation (Mayrhofer *et al*., 2005). Mechanisms uncoupling isoprene emission and photosynthesis towards the end of the ultradian cycle were not investigated. However, it is possible that larger amounts of alternative carbon sources contributed to maintain high emission rates of isoprene as photosynthesis declined (Brilli *et al*., 2007).

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**Fig. 3.** Net photosynthetic CO$_2$ assimilation ($A$, a) and isoprene emission (b) of *Eucalyptus citriodora* saplings measured in leaves exposed to increasing temperatures and 0.5, 12, and 24 h after the temperature treatment. Measurements were taken at 100, 10, and 5% fractions of transpirable soil water (FTSW), as shown in the key in a. Values are means of four plants ± 1 SEM.

**Fig. 4.** Net photosynthetic CO$_2$ assimilation ($A$), stomatal conductance ($g_s$), and isoprene emission of *Eucalyptus citriodora* saplings measured after relief of water stress. Values are means of five plants ± 1 SEM. Different letters indicate significant differences at $P < 0.05$. 
2007) because of the reduced transport conductance to CO₂ observed towards the end of the ultradian cycle (Fig. 2C). The ultradian isoprene emission kinetic recorded in these experiments was in keeping with the observations reported by Wilkinson et al. (2006), who were able to describe the whole ultradian rhythm in an experiment on palm trees which lasted about 10 hours. The current study only lasted about 8 hours and, consequently, it was not possible to measure completely the drop in isoprene emission occurring at the end of the ultradian cycle as shown by Wilkinson et al. (2006). However, if the measurements had been prolonged for more than 8 hours, it is likely that the full decline of isoprene emission would have been detected. Because the measurements were made at constant PPFD, the concurrent reduction of photosynthesis and isoprene emission may be an adaptive response to the diurnal cycle of the incoming PPFD with its typical declining trend by the end of the day. In fact, many genes associated to transcripts for components of the photosynthetic light-harvesting complex are shown to be circadian regulated and analysis of promoters for genes involved in the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway (Wiberley et al., 2009) revealed many transcription factor binding sites strictly linked to light-regulated transcriptions (Terzaghi and Cashmore, 1995). Because of the very small amount of energy required for isoprene production (Sharkey et al., 2008), a significant change in the ultradian control of isoprene emission could take place only when the severity of a stress such as drought causes a failure in the photochemistry. This would occur only when water stress is prolonged over the FTSW endpoint, when stomata are completely shut to prevent plant dehydration (Sinclair and Ludlow, 1986; Brilli et al., 2007).

As for isoprene emission, the biosynthesis of the other isoprenoids formed by *E. citriodora* leaves was only limitedly affected by water stress (Table 3). It is noteworthy that the literature shows contrasting results about water stress impact on isoprenoids, for example the foliar concentration of volatile terpenes in young *Pinus halepensis* and *Quercus ilex* saplings (Blanch et al., 2009) and the leaf terpene concentrations of four woody species growing in a Mediterranean shrubland in different seasons and years (Llusià et al., 2006). While the total amount of isoprenoids (expressed on a leaf area basis) stored in leaves remained steady, a significant variation of the components of the isoprenoid blend was observed under increasing water stress. In particular, the current results indicate that the synthesis of citronellal and citronellol, the major constituents of the isoprenoid blend stored in *E. citriodora* leaves (Olivero-Verbel et al., 2010) was consistently induced by water stress; meanwhile, the concentrations of isopulegol and caryophyllene oxide dropped dramatically. Selective up- and downregulation of constitutive isoprenoids as water stress progresses may be caused by a partial diversion of the carbon flux through the MEP/DOXP pathway to the biosynthesis of the more useful isoprenoids, as also observed in plants infested by herbivores (Brilli et al., 2009). Indeed isoprenoids stored in *Eucalyptus* spp. leaves have antioxidant properties, which also act as repellents to herbivores (Olivero-Verbel et al., 2010). Consequently, it may be speculated that isoprenoid biosynthesis in *E. citriodora* may be consistent with the ‘opportunist hypothesis’ put forward by Peñuelas and Llusíà (2004), according to which plants can efficiently change the demand of essential isoprenoids to face incoming environmental stresses.

The second experiment, performed at the onset of autumn, was designed specially to measure the optimum temperature for isoprene emission in severely stressed conditions. In contrast with the results obtained at the beginning of summer, there were no significant differences in isoprene emission measured at 30 °C between FTSW₀ and FTSW₁₀ (Fig. 3B). Seasonal variations in isoprene emission may account for these differences (Boissard et al., 2001). However, the current results again confirmed that isoprene emission is a process resistant to water stress. The optimum temperature for isoprene emission was measured at ~45 °C, and was not altered by water stress, although the optimum temperature was already reached at 40 °C when the stress was particularly severe (Fig. 3B). As the temperature-dependency of isoprene emission reflects the optimum temperature of the ISPS enzyme (Monson et al., 1992), it is concluded that water stress does not change relevantly this property of the enzyme. However, the strong inhibition of the maximum isoprene emission rate occurred when water stress was more severe (Fig. 3B) provided an indirect indication that ISPS activity is progressively impaired by water stress. Probably water stress limits isoprene emission at both transcriptional and posttranscriptional levels because the inhibition of the ISPS activity is first followed by a down-regulation of ISPS gene expression and then by a decrease of ISPS protein concentration (Brilli et al., 2007; Fortunati et al., 2008). It should be considered that exposure to a transient increase of temperature up to 45 °C in water-stressed plants represents a recurrent situation in arid areas. Therefore, the current results provide evidence that isoprene emission may have a very different temperature dependency in stress conditions than in control conditions and that the temperature optimum for isoprene emission does not acclimate in response to water stress. These findings support the opinion that the optimum temperature for isoprene emission may be shifted only after acclimation of plants to different growth temperature regimes (Monson et al., 1992; Fares et al., 2011).

Diffusive factors began to limit A in *E. citriodora* saplings at ~25% FTSW (Table 1), as shown by the progressive decline of Ci (Fig. 1), which indicates a relative higher reduction of gₜ with respect to A. Consistently with past research (Lawlor and Cornic, 2002; Centritto et al., 2003; Loreto and Centritto, 2008), metabolic limitation to A, as inferred from the increased Ci values with respect to those measured in control plants (Lawlor and Cornic, 2002), may have likely taken place only when water stress became severe (i.e. FTSW approaching zero, when there was virtually no water available to be transpired). However, these metabolic impairments may have been only transient, as demonstrated by the fast recovery of A that was completed 7 days after rewatering (Fig. 4). Similarly to isoprene emission, the kinetics of A, gₜ, and Ci in response to FTSW and rewatereing are strikingly in keeping with previous investigations on drought kinetics performed in *Populus* spp. (Brilli et al., 2007; Centritto et al., 2011a).
and S. cumini (M. Centritto and T. Mahmood, unpublished data). Thus, the inherent response of diffusional and non-diffusional limitations to photosynthesis to equivalent levels of water stress (i.e. similar percentage of transpirable soil water, irrespective of the actual soil water content) (Centritto et al., 2011a) may hold across plant functional types indicating that photosynthesis may not be limited by metabolic factors as long as water is available to support transpiration even at limited rates.

Finally, similarly to $g_s$, $g_m$ also declined as soil water deficit increased (Fig. 2). Interestingly, these results are also similar to those obtained in a study on well-watered and water-stressed Eucalyptus regnans plants (Warren, 2008). Furthermore, consistently with past studies (Centritto et al., 2003; Flexas et al., 2008; Loreto and Centritto, 2008), and as also seen in Warren’s study on E. regnans (2008), $g_m$ varied in coordinated manner with $g_s$, thus confirming that these components of CO$_2$ transport from the atmosphere carboxylation sites in chloroplasts may limit photosynthesis in a coordinated manner and to a similar extent. It is noteworthy that $g_s$ (Fig. 2B) and $g_m$ (Fig. 2C) were kept under ultra-riad control, irrespective of the water stress intensity. Such fast changes in $g_m$ may be related to the involvement of aquaporins, as they play a significant role in facilitating CO$_2$ transport in the mesophyll, rather than to structural adjustments (Kaldenhoff, 2012). As far as is known, there are no earlier data on the circadian regulation of $g_m$. However, because these measurements were made in controlled conditions under continuous PPDF of 1000 µmol m$^{-2}$ s$^{-1}$, similar measurements in more natural conditions are needed to fully understand the ultra-riad control of photosynthetic limitations.

In conclusion, the application of water stress to E. citriodora saplings demonstrates that: (a) the kinetics of photosynthetic parameters and isoprene emission as a function of the amount of soil water available to support transpiration is similar in Eucalyptus as in other plant functional types; (b) both the ultra-riad control and the temperature optimum for isoprene emission are not affected to progressive deficiency of soil water; however, water stress induces a progressive downregulation of the temperature-dependent stimulation of isoprene emission; (c) a reallocation of carbon through the MEP/DOXP pathway may result in qualitative changes of the blend of stored isoprenoids associated to the reduced isoprene emission under severe water stress; and (d) the ultra-riad trend of $g_m$ is resistant and has a similar sensitivity to that of $g_s$ to water stress. These findings are likely to be useful to refine the algorithms employed in process-based models aiming to precisely up-scale carbon assimilation and isoprenoid emissions at regional and global scales in ecosystems exposed to a forecasted warmer and drier future climate.

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